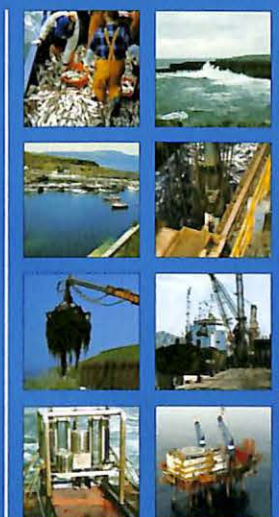


**Report on Analysis of Shellfish Samples  
for the Presence of Yessotoxins (YTX)**

**Marine Institute  
February 2001**



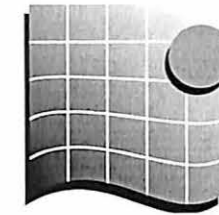
**Marine Institute**  
Ireland



**Report on Analysis of Shellfish Samples  
for the Presence of Yessotoxins (YTX)**

**Marine Institute  
February 2001**

0044  
Gerry McLundh 08 2890 417728  
John McEwen 00442890 525754  
Glen Kennedy 0044 2890 525626



*Marine Institute*  
*Foras na Mara*

To: **Minister of State Hugh Byrne T.D.**  
**Molluscan Shellfish Safety Committee**  
**S. White, Department of the Marine and Natural Resources**

31/01/01

**Re: Analysis of shellfish samples for the presence of Yessotoxins and other marine biotoxins**

**I. Sample Tests.** In order to determine the cause of the positive mouse bioassay results obtained in mussel samples from several shellfish production areas (including Bantry Bay, Kenmare Bay, Cromane and Lough Foyle), samples were sent in December 2000 and January 2001 for analysis to the laboratories listed below.

- EU Reference Laboratory on Marine Biotoxins, Vigo, Spain
- UK Reference Laboratory on Marine Biotoxins, Aberdeen, Scotland
- Italian Reference Laboratory on Marine Biotoxins, Cessnatico, Italy
- Japan Food Research Laboratories, Tokyo, Japan
- ESR, Porirua, New Zealand.

The following analyses were carried out:

- DSP Mouse Bioassay - EU Reference Laboratory, Italian Reference Laboratory
- Okadaic acid, DTXs - EU Reference Laboratory and UK Reference Laboratory
- Azaspiracid - UK Reference Laboratory
- ASP toxins (Domoic acid) - EU Reference Laboratory
- Yessotoxins - EU Reference Laboratory, Italian Reference Laboratory and Japan Food Research Laboratories

## **II. Results:**

Positive mouse bioassay results were obtained, using the Yasumoto (1978) assay, in the EU Reference Laboratory and using the Yasumoto (1984) assay in the Italian Reference Laboratory.

ASP toxins, Azaspiracid, Okadaic acid and DTXs were not detected.

Yessotoxin, homoYessotoxins and analogues of Yessotoxins were not detected in the samples sent to the EU Reference Laboratory or the Italian Reference Laboratory.



However, Prof. Yasumoto has now detected the presence of **Yessotoxin and 45-hydroxyYessotoxin** at the Japan Food Research Laboratory (copy of his results are attached). 45-hydroxyYessotoxin is a shellfish metabolite of Yessotoxin, which occurs with time as the YTX is oxidised by the shellfish. A paper by Prof. Yasumoto *et al* on this YTX toxin is also attached.

The shellfish samples in which Yessotoxin and 45-hydroxyYessotoxin were detected were taken from Bantry Bay, Roaring Water Bay (Summer 2000) and Mulroy Bay.

The difference in results from these laboratories, is primarily because the 45-hydroxy Yessotoxin reference material is only available at the Japan Food Research Laboratory and therefore was not specifically looked for at the other 2 laboratories. It may be explained by the fact that the samples sent to the 3 laboratories were taken from different locations within Ireland and at different times.

### III. Implications

If the initial detection of Yessotoxin and 45-hydroxyYessotoxin is confirmed in other samples, the Irish biotoxin management regime and the production cycles for shellfish (in particular mussels) will have to be adapted to encompass YTX.

The detection of Yessotoxins may also have implications regarding the toxin detection methods used in North of Ireland and the Republic of Ireland.

The detection of Yessotoxin and 45-hydroxyYessotoxin has contributed to our understanding of the persistent toxicity, which has led to the prolonged closures in late 2000. It also highlights the ability of the Yasumoto (1978) mouse bioassay to detect /screen a wide range of toxins of potential human health concern, where the specific toxins are not known.

YTX has been associated with the presence of several dinoflagellates, including *Gonyaulax polyedra*. Based on research by the Marine Institute, cysts of *Gonyaulax polyedra*, as well as its vegetative stages, have been found in high densities in Bantry Bay and at lower levels elsewhere. (Extract of thesis by J.Silke, 1996 is attached).

### IV. Regulatory Status of YTX

The potential human health effects of the consumption of shellfish containing Yessotoxins is currently under review by an EU Expert Working Group. A copy of the report of the meeting of this Working Group, of 24 February 1999, is attached. The Working Group has to date not established an agreed level for YTXs in shellfish. The Working Group has concluded

*"For the time being, and considering the available data and methodologies for toxin determination, YTXs should provisionally be monitored in the group of DSP toxins."*

**This means that where YTXs are detected, bay closures should be implemented in accordance with EU Directive 91/492 as it relates to DSP toxins.**

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This regulatory framework is in place for mussels in Italy, Norway, New Zealand and Japan. The only case where species specific opening is permitted is for scallops in Japan. This is possible because research has shown that YTXs are concentrated in the hepatopancreas and not in the edible parts (adductor muscle and gonad).

The Working Group also concluded "*further studies are needed in order to describe the mechanism of action of YTX, to confirm the target organs and fully to assess any potential risk of YTX.*" Such studies are currently being carried out in Norway by a team led by Prof. T. Aune, in collaboration with Prof. Yasumoto. Research work has also started in Italy and Spain.

#### V. Recommendations for consideration by MSSC.

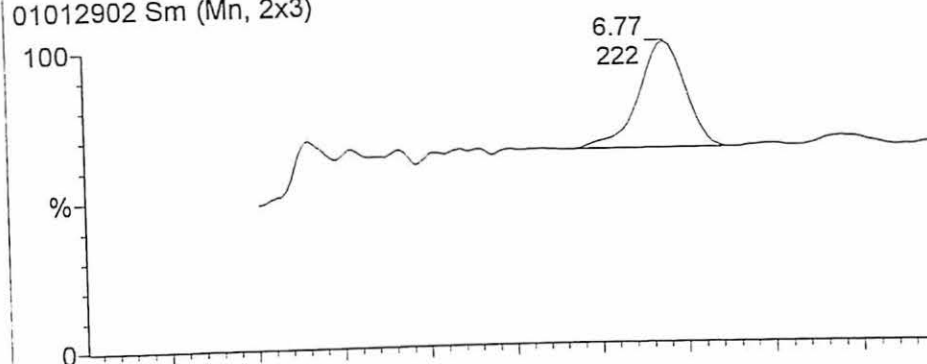
1. In the light of the new information on YTX and other toxins (AZP), provided by Prof. Yasumoto, the Marine Institute proposes that the current bioassay method (Yasumoto 1978) and the 24-hour observation period should be maintained. This is in accordance with the recommendation of the network of EU National Reference Laboratories in 1996.
2. YTXs should be included in the list of toxins to be targeted in the national chemical screening programme in 2001. LC/MS and HPLC methods have been published, but have not been used to date in Ireland.
3. *G. polyedra* should be included in the list of phytoplankton species to be targeted in the MI national phytoplankton monitoring programme in 2001
4. Further samples to be sent for YTX analysis to the EU Reference Laboratory (MI and DOMNR have already arranged for samples from Lough Foyle and Bantry Bay to be sent to Vigo).
5. The Marine Institute and other Irish agencies should work with international research partners to provide YTX standards and standard reference material. To date, YTX standards have only been produced in New Zealand. This could form part of a collaborative proposal for EU funding.
6. The Marine Institute and FSAI to collaborate with the EU Working Group on YTXs, in order to arrive as soon as possible at a regulatory limit (threshold level) for the presence of YTXs in shellfish. This recommendation will then have to be assessed by the EU Standing Veterinary Committee.

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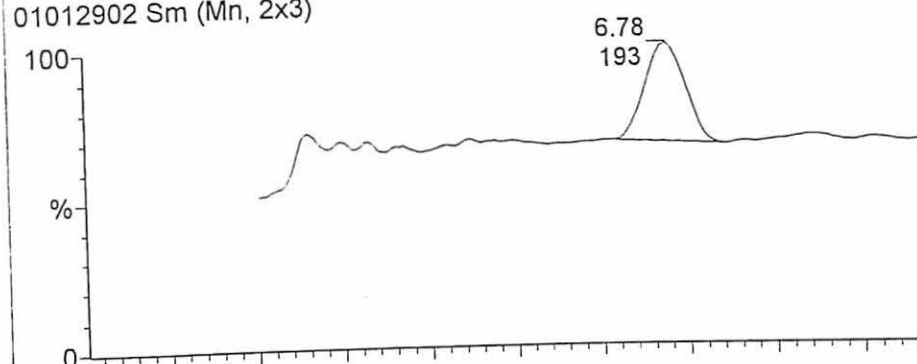
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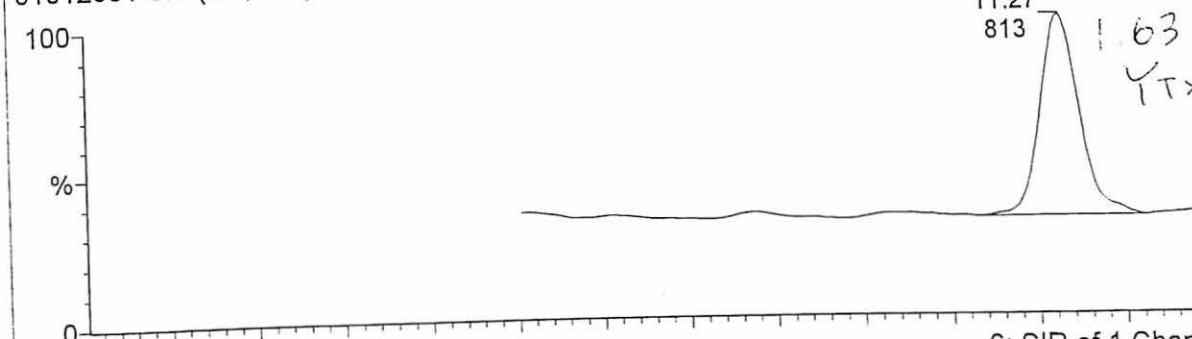
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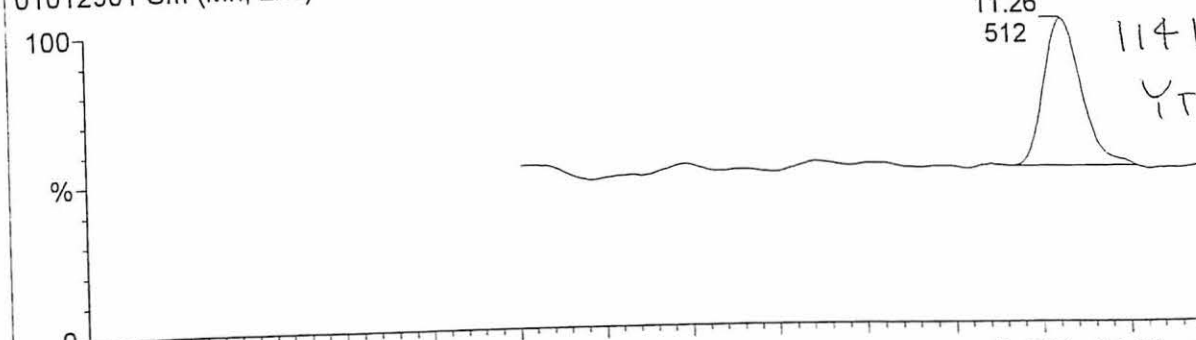
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## Toxicologic Evaluation of Yessotoxin

Hiroyuki Ogino,<sup>1</sup> Masanori Kumagai,<sup>2</sup> and Takeshi Yasumoto<sup>2\*</sup>

<sup>1</sup>Kagome Research Institute, Kagome Co., Ltd., Nishitomiya, Nasu-gun, Tochigi, Japan

<sup>2</sup>Faculty of Agriculture, Tohoku University, Tsutsumidori-Amamiya, Aoba-ku, Sendai, Japan

**ABSTRACT** Yessotoxin (YTX), originally found in association with diarrhetic shellfish poisoning (DSP), caused neither intestinal fluid accumulation nor inhibition of protein phosphatase 2A. Orally, YTX was not lethal to mice at 1.0 mg/kg. The toxin showed weak cytotoxic and antifungal activities. Neither hemolytic nor ichthyotoxic effect was observed. *Nat. Toxins* 5:255-259, 1997. © 1998 Wiley-Liss, Inc.

**Key Words:** oral toxicity; diarrheagenicity; protein phosphatase 2A inhibition; diarrhetic shellfish poisoning

### INTRODUCTION

Yessotoxin (YTX) is a disulfated toxin having contiguous ether rings aligned in a ladder-shape isolated first from scallops. Its planar structure was determined in 1987 [Murata et al., 1987], and the absolute configuration was determined recently [Satake et al., 1996; Takahashi et al., 1996] (Fig. 1). Previously the occurrence of YTX outside Japan was known only in Norway [Lee et al., 1988] and Italy [Ciminiello et al., 1997]. However, analysis by a recently developed fluorometric determination method by HPLC revealed the occurrence of YTX in mussels from Chile and New Zealand [Yasumoto and Takizawa, 1997], and a new analog homoYTX in the Adriatic Sea [Satake et al., 1997a]. Moreover, the method enabled us to identify YTX in the marine dinoflagellate *Protoceratium reticulatum* collected in New Zealand [Satake et al., 1997b]. As the dinoflagellate is commonly seen in coastal waters of many regions, more widespread occurrence of YTX in shellfish is predicted.

Though YTX often coexists with the major toxins of diarrhetic shellfish poisoning (DSP) and gives positive results when tested by the conventional mouse bioassay method for detecting DSP toxins, arguments existed as to whether or not YTX should be included in the DSP category. However, toxicologic studies necessary to assess its human health risks have been hampered due to the extremely limited availability of the toxin. In our renewed effort, we recently isolated 1.7 mg of YTX from 20 kg of toxic hepatopancreas of scallops, and carried out toxicologic studies. Because of the close structural resemblance of YTX with brevetoxin B, a well known ichthyotoxin from a red-tide organism [Lin et al., 1981], ichthyotoxicity tests were carried out. Similarly, YTX was tested for hemolytic activity to be compared with other sulfated polyether hemolysins of marine dinoflagellate origins. Taking into consideration the possible conversion of YTX to bisdesulfated YTX (dsYTX, Fig. 1) by intestinal microorganisms, dsYTX was also included in some experiments.

### MATERIALS AND METHODS

#### Materials

YTX and dinophysistoxin-1 (DTX1), the major DSP toxin in Japan, were isolated from toxic scallops. DsYTX was prepared by refluxing YTX in a dioxane/pyridine (1:1) mixture for 3 hours. Their purity was confirmed by chromatographic and spectral measurements (MS and <sup>1</sup>H NMR). Okadaic acid (OA), the major DSP toxin in Europe, and other reagents and solvents of analytical grades, were purchased from Wako Pure Chemical Industries (Osaka, Japan) and used as received, otherwise stated.

#### Mouse Lethalities Measurements

Lethality of YTX was assayed in male ddY mice (15 ± 0.5 g) by intraperitoneal injection of toxin solutions prepared with 1% Tween 60 solution. The injection volume was 1.0 ml in all cases. The assays were carried out at 4 different dose levels: 80, 100, 120, and 140 µg/kg. Three mice were used for each dose level. The observation period was 30 hours from injection.

The oral toxicity of YTX was tested in male ddY mice (20 ± 0.5 g). Test solutions of YTX were prepared as above in 1% Tween 60 solution, and 0.5 ml of the solution was administered per os (p.o.) by intubation. The doses tested were 200, 500, 750, and 1,000 µg/kg. Four to 5 mice were used for each dose level.

For comparison, 2 DSP toxins, DTX1 and pectenotoxin-2 (PTX2), were also tested for their oral toxicity. All mice were kept in fast for 24 hours before receiving the toxins.

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\*Correspondence to: Prof. Takeshi Yasumoto, Faculty of Agriculture, Tohoku University, Tsutsumidori-Amamiya, Aoba-ku, Sendai 981, Japan.

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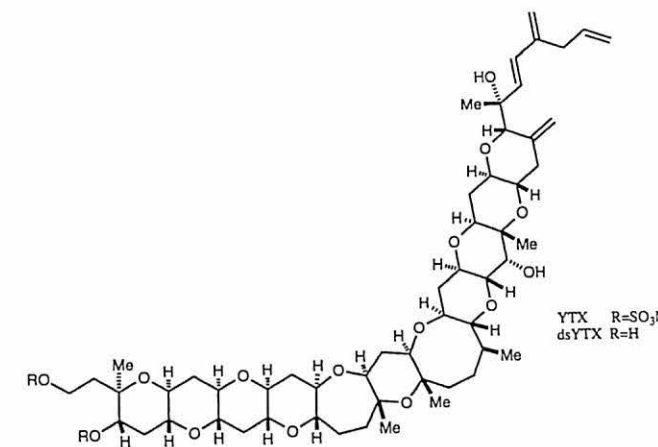


Fig. 1. Structure of yessotoxin (YTX) and dsYTX.

#### Dose-Survival Time Relationships

The relationship between the dose of YTX and the survival time of a mouse after being injected i.p. with the toxin was investigated in male ddY mice ( $15 \pm 0.5$  g). Test solutions were prepared by emulsifying YTX with 1% Tween 60 solution. The injection volume was 0.1 ml. Assays were carried out at 10 different doses: 210, 214, 267, 276, 544, 559, 563, 736, 1,067, and 1,370  $\mu\text{g/kg}$ . Due to the limit of YTX available for the test, only 1 mouse was used for each dose.

#### Diarrheagenicity Tests

Diarrheagenicity of YTX was tested following the method of Hamano et al. [1985]. Four-day-old infant mice of ddY strain were orally given (via Teflon tubing, Flon Industry, Tokyo, Japan) 0.1 ml each of YTX suspension in 1% Tween 60 solution. After the treatment, the mice were kept at  $25^\circ\text{C}$  for 4 hours and sacrificed with chloroform. The whole intestine was removed, and the fluid accumulation (FA) ratio was determined as the ratio of the weight of the intestine to that of the remaining body. Three mice were used per test and the mean value of the FA ratio was calculated. The effect of the 1% Tween 60 solution on the FA ratio was also examined. Ratios of less than 0.070 were considered negative. Those in the range of 0.070–0.090 were considered questionably positive, and those over 0.090 strongly positive. OA was used as a positive control.

#### Hemolysis Test

Hemolytic potency of YTX was tested on mouse blood cells. In a small centrifuge tube ( $8 \times 75$  mm), 50  $\mu\text{g}$  of YTX was dissolved in 30  $\mu\text{l}$  of methanol. The solution was diluted with 0.47 ml of 0.9% saline, and mixed with 0.5 ml of 2% mouse blood cell suspension in 0.9% saline. After incubation for 30 minutes at  $37^\circ\text{C}$ , the suspension was centrifuged and examined for hemolysis. As the positive control, plant saponin (Merck, Darmstadt, Germany) was used.

#### Antimicrobial Test

Growth-inhibiting activity of YTX against fungi, yeast, and bacteria was tested by a paper disk method employed in our previous study [Nagai et al., 1990]. The following organisms were used: fungi, *Aspergillus niger* and *Penicillium funiculosum*; yeast, *Candida rugosa*; Gram-positive bacteria, *Bacillus megaterium* and *Staphylococcus aureus*; and Gram-negative bacterium *Escherichia coli*. In addition to YTX, dsYTX was also tested for comparison. Amphotericin B was used as a positive standard. Test solutions were prepared by dissolving respective substances in methanol. Appropriate amounts of the test solutions were applied on filter-paper disks of 8 mm diameter. The paper disks were placed on agar plates seeded with the test microorganisms. Assays for the fungi were carried out with a yeast agar medium, those for candida with a candida medium, and those for the bacteria with a beef extract medium. The plates were incubated at  $30^\circ\text{C}$  for 2 days, and inhibition circles formed around the disks were measured.

#### Ichthyotoxicity Test

Ichthyotoxicity was carried out by using killifish, *Oryzias latipes*, as test fish. An appropriate amount of YTX was dissolved in 0.1 ml of methanol, and the solution was diluted with water to 50 ml to prepare a 1.0 or 0.5 ppm test solution. A killifish was placed in a beaker containing the test solution and observed for 24 hours. The assay was run in triplicate at each concentration. Similarly, ichthyotoxicity of dsYTX was tested at 0.5 ppm concentration.

#### Protein Phosphatase 2A Inhibition Assay

Protein phosphatase 2A (PP2A) inhibition assays were carried out essentially following the method in the literature [Tubaro et al., 1996]. The enzyme and the substrate, *p*-nitrophenyl phosphate, was purchased from Wako Pure Chemical Industries). The hydrolysis of the substrate to *p*-nitrophenol was monitored with a Microplate Autoreader (Bio-Rad Laboratories, Richmond, CA) at 405 nm. Each determination was done in triplicate.

#### Cytotoxicity Assays

The effect of YTX on cell viability was measured following the method of Scudiero et al. [1988]. The method is based on the ability of metabolically active cells to reduce MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] to a blue formazan product. Test solutions were prepared by diluting dimethyl sulfoxide (DMSO) solution of YTX with the RPMI culture medium supplemented with 10% (V/V) fetus bovine serum, 100 U penicillin G/ml, and 0.1 mg streptomycin/ml. Eight concentrations of YTX ranging from 10 to 1,000 ng/ml were prepared. The maximum concentration of DMSO in the solution was 1%. Rat Glioma C6 cells (18,000 cells) were plated in 96-well microplates in 90  $\mu\text{l}$  RPMI medium and mixed with 10  $\mu\text{l}$  of the test solution. Cells were maintained at  $37^\circ\text{C}$  in a 95% air, 5%  $\text{CO}_2$ .

TABLE I. The Lethality of YTX to Mice by I.P. and P.O. Administration

Route	Dose ( $\mu\text{g/kg}$ )	Number of mice survived	Number of mice died
i.p.	80	2	1
	100	0	3
	120	0	3
	140	0	3
p.o.	200	4	0
	500	5	0
	750	5	0
	1,000	5	0

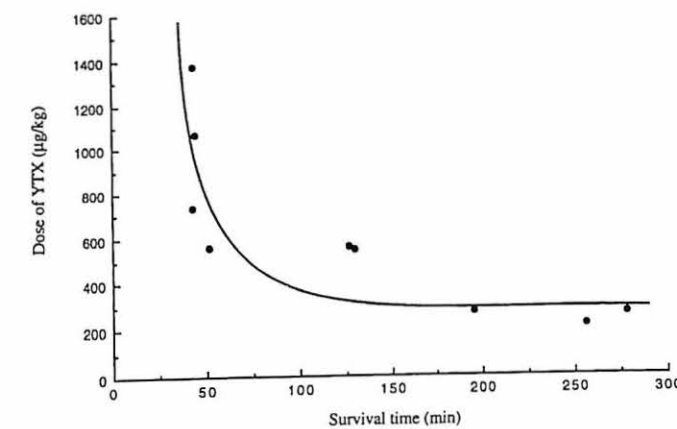


Fig. 2. Dose-survival time relationship for YTX by i.p. injection.

atmosphere. After 24 hours of contact with the test solution, 10  $\mu\text{l}$  of 0.5% MTT solution was added to each well, and 4 hours later the liquid in the wells was eliminated. Subsequently, 150  $\mu\text{l}$  of acidified isopropanol (0.04 N HCl) was added to each well to dissolve the formazan formed in the viable cells. The formazan concentration was measured with a Microplate Autoreader (Bio-Rad Laboratories, Richmond, CA) at 490 nm. OA was used as a positive standard.

#### RESULTS AND DISCUSSION

##### Toxicity to Mice

The results of the experiments to estimate the i.p. lethality of YTX are shown in Table I. All mice injected with YTX above 100  $\mu\text{g/kg}$  died. At the dose of 80  $\mu\text{g/kg}$ , 1 mouse died but 2 survived. From these data the minimum amount of YTX to kill a mouse was presumed to be in the range of 80–100  $\mu\text{g/kg}$ . Thus, mouse lethality of YTX by i.p. injection exceeded those of other DSP toxins: OA, 192  $\mu\text{g/kg}$  ( $\text{LD}_{50}$ ) [Tachibana et al., 1981] and pectenotoxin-2 (PTX2), 260  $\mu\text{g/kg}$  [Yasumoto et al., 1985].

Figure 2 shows the survival times (minutes) of mice plotted vs. the injected doses. At the dose of 563  $\mu\text{g/kg}$  or above, the survival times were in a narrow range of 40 to 50 minutes. At lower doses, death occurred within 5 hours after injection, but not later. The mouse injected with the lowest

TABLE II. The Lethality of DTX1 and PTX2 to Mice by P.O. Administration\*

Toxins	Dose ( $\mu\text{g/kg}$ )	Number of mice survived	Number of mice died	Survival time (hours)
DTX1*	100	4	1	30
	200	5	0	30<
	300	2	2	6
	400	1	3	6
PTX2*	25	3	1	24
	100	4	0	48<
	200	4	1	48
	300	3	2	6
	400	3	1	6

\*Lethal dose by i.p. injection is 200  $\mu\text{g/kg}$  for DTX1 and 260  $\mu\text{g/kg}$  for PTX2.

TABLE III. Intestinal Fluid Accumulation (FA) in Suckling Mice

Toxins	Dose ( $\mu\text{g/mouse}$ ) and response <sup>a</sup>		
	0.4	0.2	0.1
YTX	—	—	—
OA	+	+	+
DTX1	+	+	+

<sup>a</sup>+, FA ratio  $\geq 0.09$ ; —, FA ratio  $< 0.07$ .

dose of 210  $\mu\text{g/kg}$  did not die. Thus, by testing the survival times at different doses, YTX can be distinguished from OA and DTX1 which, at low doses, cause death even after 24 hours. Similarly, it will be easy to distinguish YTX from the fast-acting imine toxins found in shellfish, such as gymnodimine [Seki et al., 1995] and spirolides [Hu et al., 1995], because the survival times for these imine toxins are reportedly shorter than 20 minutes.

In contrast to the high i.p. toxicity, YTX did not kill mice by oral administration—even at 1.0 mg/kg, which is 10 times the lethal dose—by i.p. injection (Table II). No sign of intoxication was observed, and mice gained 3 g in body weight during the following 24 hours. Evidently, the oral toxicity of YTX was much less important compared with its i.p. toxicity. On the other hand, oral toxicities of DTX1 and PTX2 were comparable with their i.p. toxicities, as shown in Table II, indicating their importance as food intoxicants. In order to conclude that YTX is harmless by oral intake, however, histopathological examination of organs and tissues of animals will be necessary in the future.

Previously, Terao et al. [1990] observed that YTX did not cause intestinal fluid accumulation in adult mice by i.p. injection. In order to rule out the possible degradation or structural modification of YTX by intestinal microorganisms and in order to test YTX effect by the oral route, we examined the intestinal fluid accumulation in infant mice by intubation. The results are shown in Table III. YTX did not cause intestinal fluid accumulation at the dose of 0.4  $\mu\text{g/mouse}$ , while reference OA and DTX1 did at 0.1

TABLE IV. Antimicrobial Activity of YTX and dsYTX by the Paper Disk Method

Test microorganisms	YTX ( $\mu\text{g}/\text{disk}$ )		dsYTX ( $\mu\text{g}/\text{disk}$ )	
	10	50	10	50
<i>P. funiculosus</i>	— <sup>a</sup>	+	+	+
<i>A. niger</i>	+	+	+	+
<i>C. rugosa</i>	NT <sup>a</sup>	+	+	+
<i>B. megaterium</i>	—	—	—	—
<i>S. aureus</i>	—	—	—	—
<i>E. coli</i>	—	—	—	—

<sup>a</sup>+, positive; —, negative; NT, not tested.

$\mu\text{g}/\text{mouse}$ . The infant mice administered higher doses of YTX died, implying their higher susceptibility compared to the adults.

#### Hemolytic Property

The two sulfate groups at 1 terminus of the hydrophobic polyether skeleton were suggested to give the molecule an amphiphilic property. Thus, hemolytic activity of YTX was expected, as is the case with amphidinol [Satake et al., 1991] and maitotoxin [Yasumoto and Murata, 1993]. At the concentration tested (50 ppm), however, YTX did not cause hemolysis. Under the same condition, the plant saponin caused complete hemolysis at 10 ppm concentration.

#### Ichthyotoxic Property

Because of the close structural resemblance of YTX with brevetoxin B (lethal concentration to zebra fish; 0.016 ppm or 18 nM by Lin et al. [1981]), we were interested in the effect of YTX on fish. However, none of the fish exposed to 1 ppm (840 nM) or 0.5 ppm (420 nM) solution of YTX died within 24 hours. Three fish exposed to 0.5 ppm (510 nM) solution of dsYTX did die, but only after 6 hours. It may be concluded, therefore, that ichthyotoxicity of YTX is insignificant, if there is any.

#### Antimicrobial Property

Table IV shows the result of the antimicrobial tests carried out on YTX and dsYTX. The 2 compounds did not inhibit bacterial growth, but did inhibit growth of fungi and yeast. DsYTX was more potent than YTX. Their potencies (10–50  $\mu\text{g}/\text{disk}$ ) were comparable with that of amphotericin B (20  $\mu\text{g}/\text{disk}$ ).

#### Inhibition of Protein Phosphatase 2A (PP2A)

As shown in Figure 3, YTX inhibited hydrolysis of *p*-nitrophenyl phosphate by PP2A. The concentration required to reduce the enzymatic activity by 50% ( $\text{IC}_{50}$ ) was 0.36 mg/ml. The potency was lower than that of OA by 4 orders of magnitude. Hence, interference by YTX coexisting with OA in shellfish can be disregarded in the enzyme inhibition assay for OA or DTX1.

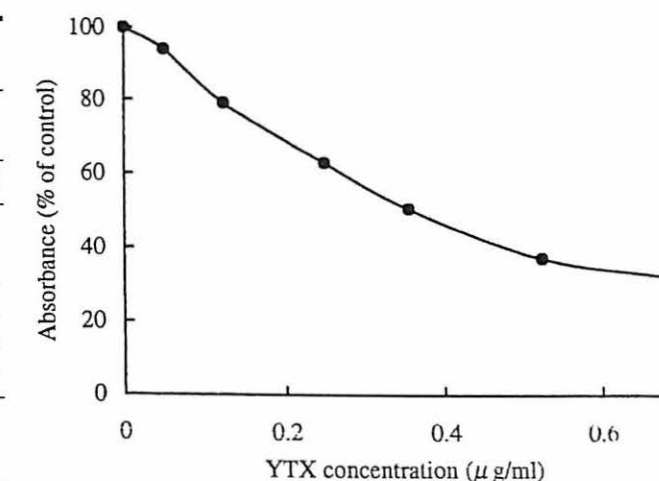


Fig. 3. Inhibitory effect of YTX on PP2A activity. Each value represents the mean of 3 experiments. Standard deviations are not shown because they were less than 5% of the mean values.

#### Cytotoxicity

Previously, cytotoxic effect of YTX on hepatocytes was observed [Aune, 1988]. Our attempt to define the accurate potency by using MTT did not succeed, because cells were detached from the plates when exposed to YTX. Although the cell detachment occurred at as low concentration as 10 ng/ml, formation of the blue formazan in the cells collected by centrifugation was observed even in 8.4  $\mu\text{M}$  solution of YTX. On the other hand, OA reduced the formazan formation to 50% at the concentration of 10.5 ng/ml (13 nM). Thus, as far as the inhibition of the formazan formation is concerned, YTX was less toxic than OA by 3 orders of magnitude.

The present study revealed that YTX is more toxic than OA when compared by i.p. injection. However, mice did not die when YTX was orally administered at the maximum dose of 1 mg/kg. It caused neither intestinal fluid accumulation in infant mice by intubation nor inhibition of PP2A. Thus, we may reasonably conclude that YTX is much less hazardous than OA or DTX1 to human health and that YTX should not be included in the DSP category. Nevertheless, the cell detachment and the lethal effect on infant mice as observed in this study should be kept in mind, together with the damage to the heart muscle reported by Terao et al. [1990]. In order to distinguish YTX from OA and DTX1, the recently developed fluorometric HPLC method for YTX [Yasumoto and Takizawa, 1997] will be useful. Because the small amount of YTX prevented us from carrying out all the experiments necessary for toxicological evaluation, we are continuing effort to accumulate the toxin for further studies.

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## Two New Analogs of Yessotoxin, Homoyessotoxin and 45-Hydroxyhomoyessotoxin, Isolated From Mussels of the Adriatic Sea

Masayuki Satake,<sup>1</sup> Aurelia Tubaro,<sup>2</sup> Jong-Soo Lee,<sup>3</sup> and Takeshi Yasumoto<sup>1\*</sup>

<sup>1</sup>Faculty of Agriculture, Tohoku University, Tsutsumidori-Amamiya, Aoba-ku, Sendai, Japan

<sup>2</sup>Dipartimento Di Scienze Biomediche, University of Trieste, Via Giorgieri 9 Trieste, Italy

<sup>3</sup>Department of Marine Food Science and Technology, Gyeongsang National University, Inpyeong-dong, Tongyeong, Kyeongnam, Korea

**ABSTRACT** Two new analogs of yessotoxin (YTX), homoYTX and 45-hydroxyhomoYTX, were isolated from the digestive glands of mussels cultured in the Adriatic coast of Italy. Their structures were determined by MS and NMR spectroscopies. *Nat. Toxins* 5:107-110, 1997. © 1997 Wiley-Liss, Inc.

**Key Words:** diarrhetic shellfish poisoning; yessotoxin; YTX; homoyessotoxin; 45-hydroxyhomoyessotoxin; adriatic mussels

### INTRODUCTION

Yessotoxin (YTX) and its analogs, 45-hydroxyYTX and 45,46,47-trinorYTX, were first isolated in Japan from the scallop, *Patinopecten yessoensis*. Their structures including the absolute stereochemistry were elucidated by NMR spectroscopy and by negative FAB MS/MS experiments except for the configuration of C45-OH [Murata et al., 1987; Naoki et al., 1993; Satake et al., 1996; Takahashi et al., 1996]. Later, YTX was detected in Norway [Lee et al., 1988] and more recently in Italy [Ciminiello et al., 1997]. YTX often coexists with diarrhetic shellfish toxins (DST) but is undetectable either by the fluorometric HPLC method developed for DST [Lee et al., 1987] or by the protein phosphatase 2A inhibition assay developed for okadaic acid [Tubaro et al., 1996]. YTX shows a potent mouse lethality (0.1 mg/kg) when administered intraperitoneally, but the toxicologic effects to human health are virtually left unknown. Thus, its contamination in bivalves causes serious damages to shellfish industries that are regulated by mouse assay results. During the course of mussel toxicity surveys, significant discrepancies were observed between the mouse assay results and those obtained either by the fluorometric HPLC method [Zhao et al., 1994] or by the protein phosphatase inhibition assay [Tubaro et al., 1996]. To explain for the discrepancies, we isolated new toxic agents. In this article, we report the structural determination of two new analogs of YTX, homoYTX and 45-hydroxyhomoYTX, isolated as the major toxins in mussels from the northern Adriatic Sea.

### MATERIALS AND METHODS

#### Isolation

HomoYTX and 45-hydroxyhomoYTX were isolated from the digestive glands (1.3 kg) of mussels, *Mytilus galloprovincialis*, collected in August 1996 from one sampling site located along the Emilia Romagna coasts of Italy. The digestive glands were found toxic by the mouse bioassay method for DSP [Yasumoto et al., 1978] but contained only low levels of okadaic acid as determined by the enzyme inhibition assay [Tubaro et al., 1996]. HomoYTX and its hydroxylated analog were isolated by following the procedures reported previously [Murata et al., 1987]. Steamed digestive glands were extracted with acetone 2 times. After evaporating acetone, the extract was partitioned between MeOH-H<sub>2</sub>O (8:2) and hexane. Toxic residue obtained in the MeOH-H<sub>2</sub>O (8:2) layer was next partitioned between BuOH and H<sub>2</sub>O. The butanol fraction was evaporated, dissolved in CHCl<sub>3</sub>-MeOH (1:1), and passed through an alumina column (ICN Biomedicals). The column was washed with CHCl<sub>3</sub>-MeOH (1:1) and MeOH, and finally YTXs were eluted with 1% NH<sub>4</sub>OH-MeOH (1:1). The toxic residue was chromatographed on a silica gel column (Merck) with CHCl<sub>3</sub>-MeOH

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\*Correspondence to: Takeshi Yasumoto, Faculty of Agriculture, Tohoku University, Tsutsumidori-Amamiya, Aoba-ku, Sendai, 981 Japan. E-mail: satake@biochem.tohoku.ac.jp

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TABLE I. <sup>1</sup>H NMR Chemical Shifts (Δ) of YTX, HomoYTX, 45-HydroxyYTX, and 45-HydroxyhomoYTX

Position	YTX <sup>a</sup>	HomoYTX <sup>a</sup>	45-HydroxyYTX <sup>b</sup>	45-Hydroxy-homoYTX <sup>b</sup>	Position	YTX <sup>a</sup>	HomoYTX <sup>a</sup>	45-HydroxyYTX <sup>b</sup>	45-Hydroxy-homoYTX <sup>b</sup>
1	4.33	4.50	4.21	3.96	26	1.79	1.79	1.72	1.68
	5.02	4.50	4.21	4.00	27	2.90	2.90	2.78	2.78
2	2.02	1.92	1.92	1.78	28	3.43	3.44	3.30	2.28
	2.64	2.26	2.22	1.83	29	1.87	1.86	1.53	1.50
2a		2.03		1.61		2.58	2.58	2.25	2.25
		2.21		1.78	30	4.14	4.13	3.59	3.60
4	5.01	4.88	4.21	4.21	31	3.44	3.44	3.19	3.15
5	2.01	2.09	1.74	1.70	32	4.36	4.36	3.83	3.82
	3.19	3.28	2.58	2.56	34	4.34	4.33	3.78	3.75
6	2.79	2.95	3.05	3.00	35	1.92	1.92	1.49	1.46
7	3.42	3.42	3.31	3.33		2.52	2.51	2.10	2.09
8	1.46	1.57	1.42	1.39	36	4.74	4.74	4.05	4.02
	2.31	2.36	2.18	2.16	37	3.79	3.79	3.40	3.36
9	3.20	3.22	3.17	3.12	38	2.65	2.66	2.42	2.41
10	3.09	3.18	3.15	3.11		3.26	3.27	2.73	2.70
11	1.70	1.70	1.30	1.38	40	4.21	4.21	3.88	3.86
	2.58	2.58	2.27	2.22	42	6.12	6.12	6.23	6.23
12	3.19	3.11	3.03	3.01	43	6.67	6.67	5.99	5.96
13	3.24	3.25	3.08	3.06	45	3.01	3.01	4.81	4.78
14	1.77	1.73	1.42	1.42		3.01	3.01		
	2.59	2.59	2.30	2.28	46	5.95	5.95	5.92	5.92
15	3.45	3.46	3.35	3.31	47	5.08	5.08	5.18	5.18
16	3.32	3.32	3.24	3.21		5.11	5.11	5.32	5.32
17	1.99	1.97	1.81	1.78	48	1.23	1.21	1.28	1.19
	2.05	2.08	1.96	1.93	49	1.33	1.34	1.26	1.26
18	1.89	1.89	1.81	1.79	50	1.28	1.28	1.16	1.16
	1.98	1.99	1.89	1.86	51	1.14	1.13	1.03	1.02
20	3.46	3.46	3.41	3.41	52	1.38	1.38	1.21	1.22
21	1.91	1.91	1.75	1.72	53	4.92	4.92	4.81	4.81
	2.03	2.05	1.96	1.91		5.08	5.08	5.02	5.00
22	3.50	3.51	3.50	3.49	54	1.75	1.75	1.38	1.38
24	1.59	1.60	1.51	1.47	55	5.01	5.01	5.15	5.13
	1.90	1.91	1.77	1.73		5.08	5.08	5.24	5.22
25	1.42	1.43	1.52	1.46					
	1.75	1.77	1.80	1.78					

<sup>a</sup><sup>1</sup>H NMR spectra were measured in C<sub>5</sub>D<sub>5</sub>N. C<sub>5</sub>D<sub>5</sub>N taken as 7.58.

<sup>b</sup><sup>1</sup>H NMR spectra were measured in CD<sub>3</sub>OD. CD<sub>3</sub>OD taken as 3.31.

(9:1) and CHCl<sub>3</sub>-MeOH (1:1) to obtain toxins in the second eluate. The toxins were dissolved in MeOH-H<sub>2</sub>O (3:7) and passed through a silica gel ODS-Q3 column (Fuji gel, 10 × 270 mm) equilibrated with the same solvent. The column was then washed stepwise with MeOH-H<sub>2</sub>O (3:7, 1:1, 8:2) and MeOH in this order. The toxins eluted in the MeOH-H<sub>2</sub>O (8:2) fraction were further purified on a Toyo-pearl HW-40 column (Tosoh) with MeOH-H<sub>2</sub>O (1:1). The presence of YTXs in the eluates was checked by TLC (silica gel 60, CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O 30:10:1) and by monitoring ultraviolet absorption at 230 nm. Further HPLC purification on a Develosil ODS-7 column (Nomura Chemicals, 10 × 250 mm) with MeOH:MeCN:H<sub>2</sub>O (2:1:2) led to separation between 45-hydroxyhomoYTX and homoYTX.

#### MS and NMR Spectra Measurements

The electron spray ionization (ESI) mass spectra were measured with a Finnigan mat TSQ-700 spectrometer. NMR spectra were measured with a Varian Unity INOVA 600 spectrometer at 20°C in C<sub>5</sub>D<sub>5</sub>N or CD<sub>3</sub>OD.

#### RESULTS AND DISCUSSIONS

From 1.3 kg of the digestive glands, homoYTX and 45-hydroxyhomoYTX were obtained in 2.4-mg and 1.4-mg yields, respectively. YTX was not detected in this sample. A good separation between homoYTX and 45-hydroxyhomoYTX was achieved on the final ODS column: elution volumes for homoYTX and 45-hydroxyhomoYTX were 13





ESI-MS gave an ion peak corresponding to (M-Na)<sup>+</sup> of homoYTX at *m/z* 1,177. Thus, homoYTX was indicated to be 14 mass units larger than YTX. Most probably, homoYTX has an extra methylene or methyl to the structure of YTX. The structural elucidation of homoYTX was mainly carried out by comparing the <sup>1</sup>H-NMR data between homoYTX and YTX. <sup>1</sup>H-NMR signal assignments were done by <sup>1</sup>H-<sup>1</sup>H COSY measurements in C<sub>5</sub>D<sub>5</sub>N at 20°C (Table I). Proton connectivities from H-4 to H<sub>2</sub>-18, H-20 to H-22, H<sub>2</sub>-24 to H-32, H-34 to H-38, H-40, H-42 to H-43, and H<sub>2</sub>-45 to H<sub>2</sub>-47 in homoYTX were identical with those in YTX. Chemical shifts and coupling constants of protons from H-7 to H<sub>2</sub>-18, H-20 to H-22, H<sub>2</sub>-24 to H-32, H-34 to H<sub>2</sub>-38, H-40, H-42 to H-43, and H<sub>2</sub>-45 to H<sub>2</sub>-47, and these of methyls except for Me-48, and three exomethylenes (C-47, C-53, C-55) were virtually unchanged between homoYTX and YTX. <sup>1</sup>H connectivities from H<sub>2</sub>-1 to H<sub>2</sub>-2a were easily determined by <sup>1</sup>H-<sup>1</sup>H COSY. Connectivity around C-3 was determined by HMBC correlations from Me-48 to C-2a and C-4. NOE correlations observed between angular protons, between angular protons and angular methyls (H-7/Me-48, H-37/Me-52), and between angular methyls (Me-49/Me-50) allowed us to determine not only the positions of the ether linkages but also the stereostructure of the whole molecule of homoYTX.

Mouse assay results obtained on homoYTX and 45-hydroxyhomoYTX suggested that their lethalities were comparable with those of YTX (0.1 mg/kg) and 45-hydroxyYTX (0.5 mg/kg), respectively. More than 70% of the mouse lethality of the digestive glands was accounted for by homoYTX and 45-hydroxyhomoYTX combined. Interestingly, mussels harvested at the same location in the previous year, 1995, contained neither homoYTX nor 45-hydroxyhomoYTX. The composition and the relative abundance of YTXs in bivalves seem to vary regionally, seasonally, and annually as observed on other DSP toxins. Presumably, YTX and homoYTX were produced by different plankton species, and the different composition between 1995 and 1996 arose from the change in the plankton population. Implication of the YTXs in human illness has not been known. However, we have to be vigilant on the occurrence of these toxins until their potential risks to human health are better understood. Details of the toxicologic effects of the YTXs will be published elsewhere.

## ACKNOWLEDGMENTS

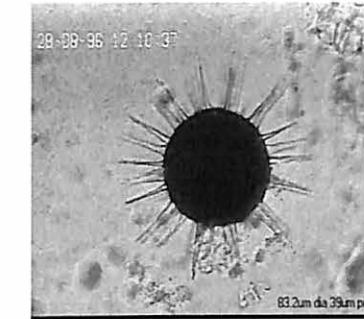
This work was supported by a grant-in-aid from the Ministry of Education, Science, Sports and Culture, Japan (07102002 and 06240101) and also by a grant of University of Trieste, Italy (MURST 60%).

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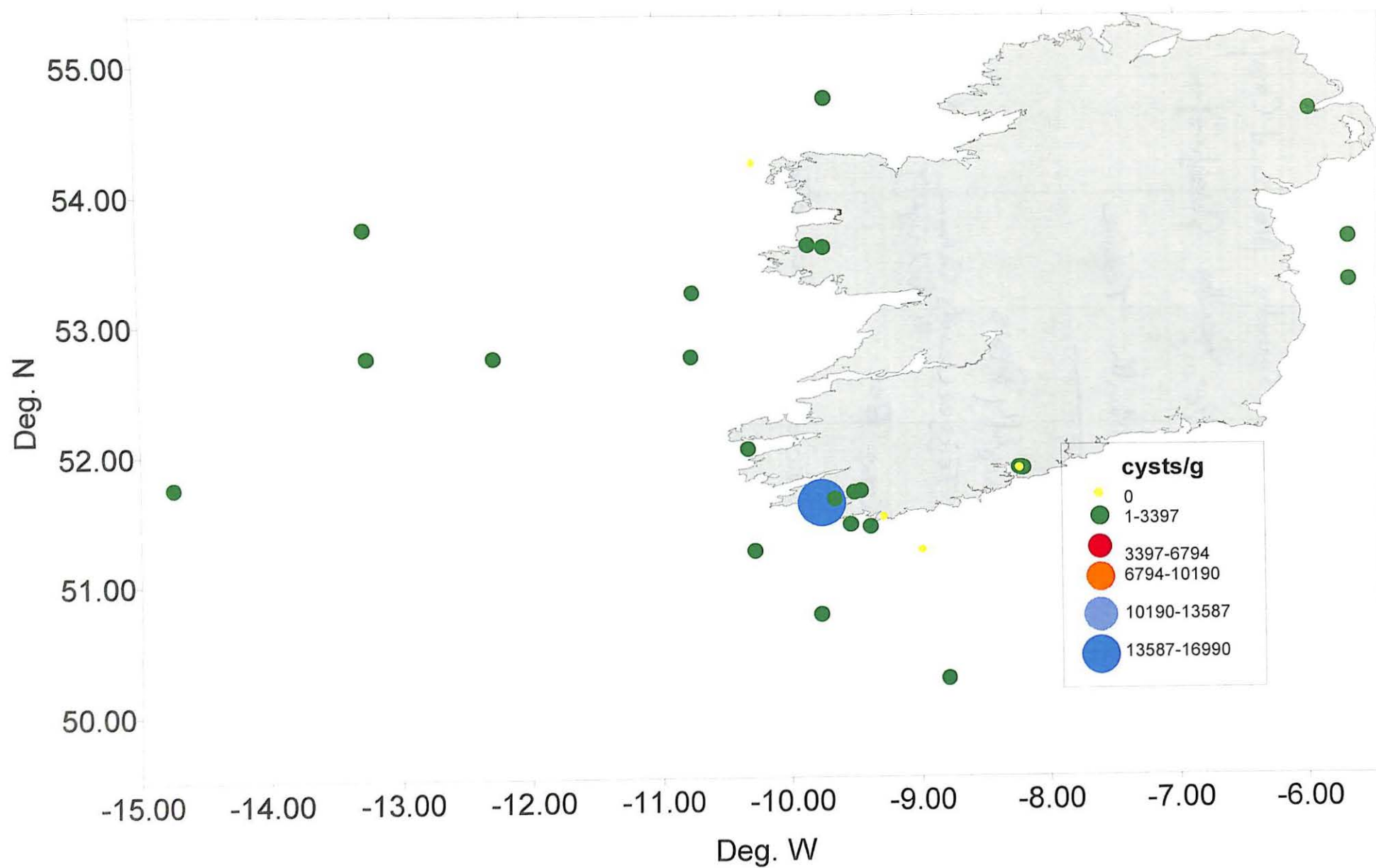
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*Gonyaulax polyedra* (syn: *Lingulodinium polyedra*)  
[Palaeontological taxon: *Lingulodinium machaeorophorum*]



*This cyst had a two layered wall (body 35-50µm in diameter) bearing numerous spines that were variable in length (3-17µm). The spines tapered from a large base and some variety of form was seen even on single specimens. Live cysts had a red accumulation body within the protoplasm. These were the most widely distributed cysts in this study and were found at most of the sampling locations, this cyst also had the highest concentration of any species (~17000/g in Bantry Bay). The distribution is mapped in Figure 3.1d. The cysts were found in 81.3% of surface sediment samples examined. Blooms of the motile stages were also recorded in Bantry Bay during the course of this survey. This species was found to have a strong neretic tendency, despite the designation of the cyst as a estuarine species by Wall et al. (1977). This species has been reported to produce Yessotoxin, in Japanese and other waters although the toxicity of the species is somewhat under discussion (Lewis and Hallet 1997).*



Distribution of *Gonyaulax polyedra* (*Lingulodinium polydrum*)  
cysts at stations 9501 - 9526 and 9601 - 9606

Monitoring of Beds is monitored by

(a) Boundary

(b) Species

A/ Microbiologist - after 3 months provisional Classification  
- 12 months Classification

B/ Biotoxin - 1 per Month Tissue

C/ Water - Fortnightly Basis  
Trigger Levels enhance Fresh test  
to fortnightly  
1 pos. close Bed

D/ Heavy Metals / Pesticides / Radionuclides

Monthly - cost factors + lab capability

Little Harvesting on North Side

G McCurdy

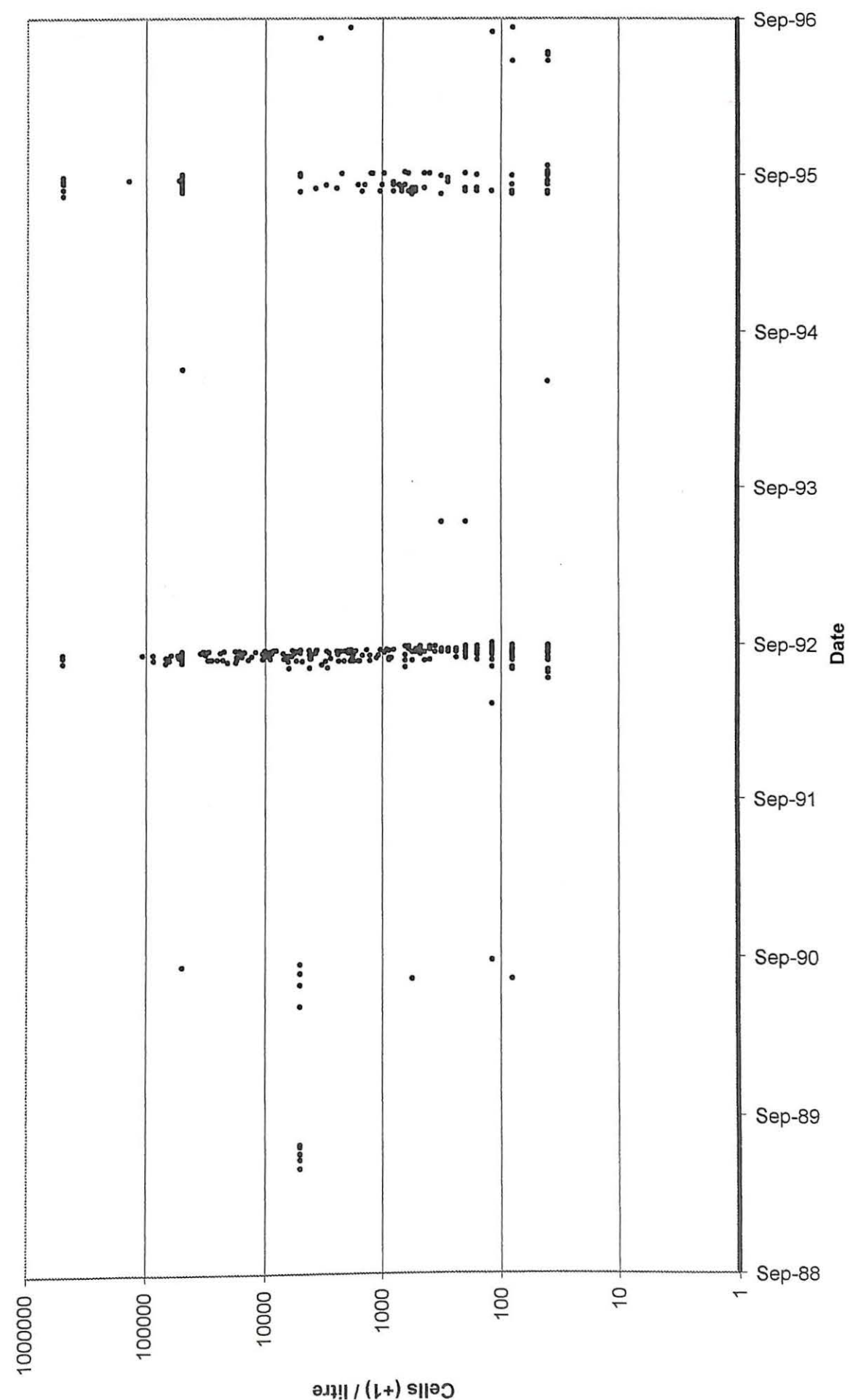


Fig 3.10: Time Series 1988 -1997 *Gonyaulax polyedra* in weekly phytoplankton samples from Bantry area.



①

Relis is Meeting Report on YTx Legislative?

Marisa

- Discussed @ 2000 NRC meeting
- Another meeting is called (1-2 months)

Norwegian work → Single Administration } 3 months  
Repeat Administration }

Norwegians have applied 100 µg for internal consumption

European Community Reference Laboratory on Marine Biotoxins  
DG VI of the European Commission

Brussels 24-2-99

MEETING REPORT

Working Group on Toxicology of Yessotoxins, Pectenotoxins and the Okadaic Acid group toxins.

A- INTRODUCTION:

At the initiative of the European Community Reference Laboratory on Marine Biotoxins and the DG VI of the European Commission, a meeting of a *small specialised working group* was held in Brussels on 22 February 1999, on the 'Toxicology of Yessotoxins, Pectenotoxins and the Okadaic Acid group toxins'.

The meeting was chaired by Mrs. Fernández from the European Community Reference Laboratory on Marine Biotoxins. The Panel was formed by a group of four experts in the field of toxicology, Dr. Cabral (Portugal), Dr. Dayan (United Kingdom), Dr. Diogène (France) and Dr. Speijers (Netherlands) in order to respond to specific questions raised on the agenda regarding the toxic potential of these toxins. Mrs. Fernández and Dr. Aune (Norway), experts in the field of diarrheagenic toxins, gave assistance to the Panel. All participants to the meeting had previously received from the European Community Reference Laboratory on Marine Biotoxins an extended bibliography on the subject.

The agenda of the meeting was the following

1. Brief state of the art

2. **Yessotoxins:**

Assessment of the experts on:

- i) are these compounds hazards to human health?
- ii) at what concentrations can they be considered harmful?
- iii) can safety limits be defined?

II. If the data is insufficient to reach a conclusion, what further studies are required  
Conclusions and proposals.

3. **Pectenotoxins:**

Assessment of the experts on:

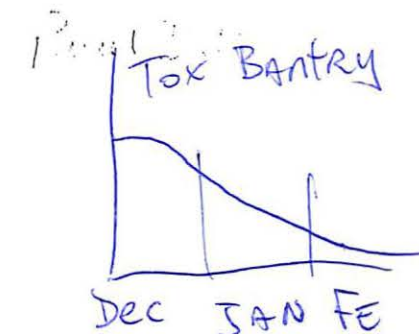
- i) are these compounds hazards to human health?
- ii) at what concentrations can they be considered harmful?
- iii) can safety limits be defined?

II. If the data is insufficient to reach a conclusion, what further studies are required  
Conclusions and proposals.

4. **Okadaic acid:**

Assessment of the experts on:

- i) Examination of data on tumour promotion
- ii) Is there sufficient information to determine if this is a potential long term hazard for consumers?
- iii) Can safety limits be set?
- iiii) Does further work have to be done on this aspect?





(10)

Whitson Report of YTX working group

Conclusion YTX should be monitored in group of DSP Toxins.

Yasumoto + Aune work is advanced

Working group to reconvene at early opportunity to look at this work

→ MF to get onto commission to get meeting organized

Yasumoto → Yessotoxin → 45 OH YTX

↓  
IP injection is 1/2 Toxic as YTX

→ Yessotoxin included in DSP complex?

Structure is different

Not PP2A inhibitor

Doesn't produce diarrhoea

→ Yessotoxin produces Azaspiracid like result on cytotoxicity

→ Azg Not very unstable in Jap experiments in Ammonia

PTX → Toxic Diarrhoea Liver toxic Kidney.

→ Seco Toxin open Ring PTX No cytotoxicity! No Bioassay +ve!

→ in scallops PTX convert to OH PTX (Not open Ringed Seco PTX)

→ Confirmation (if the toxicity of Seco Acid is necessary.)

## B- MEETING PRESENTATIONS AND DISCUSSIONS:

The meeting was opened by Mrs. Laso, from the DG VI of the European Commission, who welcomed the participants and noticed the European Commission concern for diarrheagenic marine toxins, especially regarding the different issues in the agenda.

1. Mrs. Fernández summarized the toxicological properties of the toxins of the OA group, Yessotoxins and Pectenotoxins, gave brief introductions to related subjects, and informed about the agreements on DSP toxins reached by the network of European Reference Laboratories. Dr. Aune presented an outline of experimental results obtained in his laboratory regarding preliminary toxicological studies on Yessotoxins.

2. The Panel briefly discussed the safety limit for diarrheagenic toxins under current regulations and noted that although different factors, such as the amount of ingested food, the variability of the mouse bioassay and the interaction with additional toxins could be unpredictable, the current level of protection agreed by the group of EU-NRLs was considered to be provisionally satisfactory. Recent episodes of diarrheagenic intoxications in Europe were matter of concern.

*This general view was based on reassurance given by representatives of NRLs that the variety of assay techniques they used appeared to detect most of the well known toxins, although new toxic substances were still being recognised, e.g. Killarey Bay toxin (Azaspiracid).*

3. The tumor promotion activity of the Okadaic Acid (OA) group toxins was recognized but according to the available data this was not considered a risk for Public Health in regard to the consumption of marine bivalves. A genotoxic potential of OA had been ruled out and only the diarrheagenic acute effect was considered to be of importance for safety regulations.

*The lack of concern about the promoting activity of OA was based on the fact that this activity had only been shown in animal experiments employing high topical doses over a long period, that there was no evidence to support its activity after oral administration, that there was no good evidence of oral promoters in man or animals affecting the development of tumours in tissues relevant to humans, and that the amounts of OA found in marine bivalves consumed by humans was far below the level at which actions had been detected in rodents.*

4. The Panel noted that YTX does not produce diarrhoea. After intraperitoneal injection of Yessotoxins (YTX) pathological effects on the heart and liver have been shown. The Panel evaluated experimental studies based on the oral intake of YTX by the mouse, which have suggested some toxicity of extremely high doses to the gastro-intestinal tract.

The Panel commented that little information in this field existed and agreed on the need for more studies to evaluate the risk of YTX. The reason for continuing to monitor YTX for the present lay in our limited knowledge of its toxicity. The role of the mouse bioassay or other techniques to detect YTX required careful evaluation because the key actions of YTX after IP injection in mice [hepatic and cardiac damage] differed from the diarrheagenic effect for which the test had been standardised.

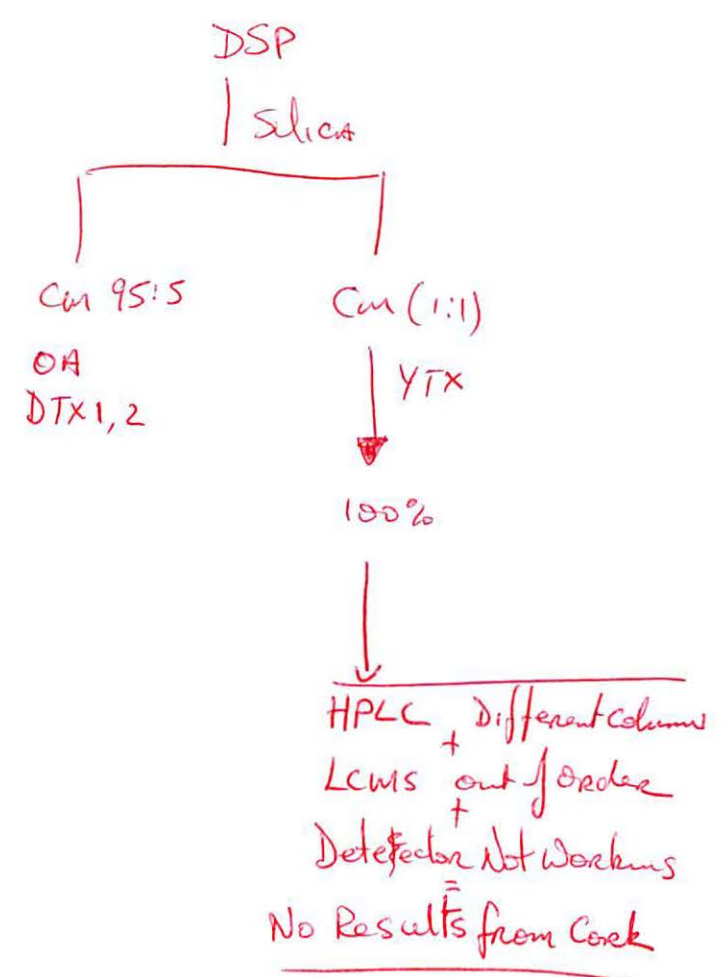
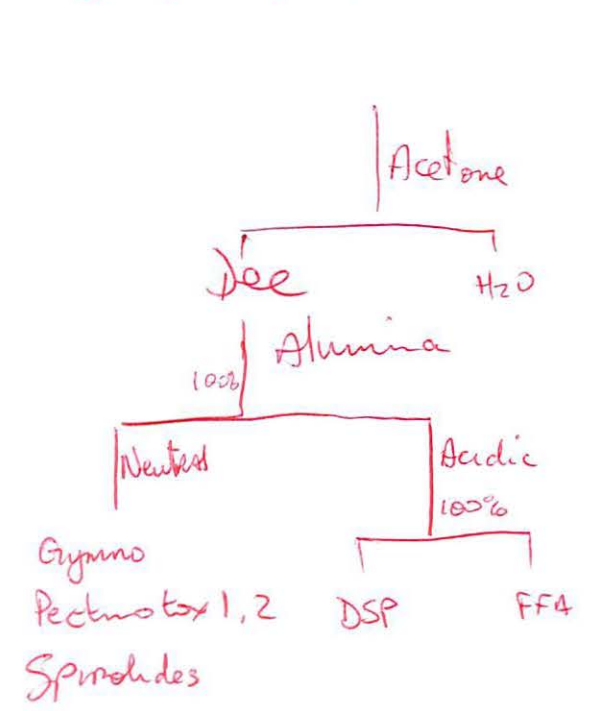


(11)

# Yessotoxin

## Assumptions

- Lot 1 ~~Manukau~~ - ~~Blue~~ RWB YTX
- Lot 2 ~~Manukau~~ - Mulkey 450H YTX 0.4 - 0.45 ppm
- 3 Bantay Bay - JAN 2001



The purpose of future experiments would be to investigate further the dose-response relationship and nature and causes of the limited toxic actions reported in mice after oral dosing of YTX. In this way, it would be possible to show whether there was any toxic risk to man from eating contaminated shellfish and what was the best way to detect it. It was recognised that the present mouse bioassay technique was not designed to measure YTXs, particularly as they do not cause diarrhea. The relative value of a simple lethality test, as in the present mouse bioassay, or a more sensitive toxicological indicator, could be determined if the evidence indicated a risk to humans.

Although the organisms responsible for the production of YTX have been identified in New Zealand (YTX- *Protoceratium reticulatum*) and in Italian waters (homoYTX- net haul dominated by *Lingulodinium polyedrum*), only the cultures of the former organism from New Zealand produce toxins in culture. The recent availability of a YTX standard could stimulate future research on the toxicity of the toxin to better understand its real risk to public health.

Until now, and considering the available data and methodologies for toxin determination, YTXs have been monitored in the group of DSP toxins. This relies on the mouse bioassay, which had been devised for the detection of other toxins with different chemical properties and biological activities. If it was shown that YTXs represented a risk to human health, a suitable method for their detection would have to be agreed.

The justification for continuing the provisional monitoring YTXs until new data became available is the limited information available about their oral toxicity, especially in humans. Their inclusion amongst the group of 'DSPs' is not appropriate in view of their known toxic properties. Agreement about how to monitor YTX is required.

5. PTX is recognized to be hepatotoxic after IP injection. It is difficult to assess its oral toxicity from current data. The lack of a standard for PTX and the impossibility of culturing the producing organism are major problems in further experimentation. Although PTX co-occurs in phytoplankton and shellfish with toxins of the OA group and has been considered to be a minor toxin, PTX has occasionally been reported to be the dominant toxin.

6. The Panel was aware of the importance of the technical details of the mouse bioassay in defining what toxins were detected. However, the panel did not extensively discuss the bioassay, or alternative detection methods, as these were considered too important subjects of discussion which were beyond the agenda of the present meeting and that will require specific attention.

After summarizing the meeting presentations and discussions, the Panel agreed the following conclusions in order to respond to the specific questions of the agenda.



No Effect Level  
Single Administration  
Repeat Administration  
[ >1 mg/kg no effect on Cardiac tissue  
High Levels Causes damage

\* Denis/Liz comment  
2000 overall to give a brief  
overview from a industry  
perspective.

## C - CONCLUSIONS:

### I - Yessotoxins

Toxicological studies on the effects of Yessotoxins (YTX) demonstrate that these toxins do not produce diarrhea *but that IP injection in mice had caused* pathogenic effects in the heart and liver. Preliminary toxicological data after oral exposure to YTX *showed that some effects on the mucosa of the GI tract were produced after much higher oral doses of YTX than after IP administration. It was recognised that the limited information from man did not indicate that YTXs caused diarrhoea in people.*

Oral intoxication *and pharmacokinetic investigations in animals* are needed in order to describe the mechanism of action of YTX, to *confirm the target organs and fully* to assess *any* potential risk of YTX. *It would be helpful to collect clinical information about instances of human poisoning. From those results it would be possible to assess the value of monitoring YTXs in marine bivalves, leaving others to consider the most suitable assay technique.*

Noting the recent availability of YTX standard and the possibility of growing the YTX producing organisms, new studies on the effects of YTX, as well as the development of determination methodologies for YTX are expected *to be done* in the near future.

For the time being, and considering the available data and methodologies for toxin determination, YTXs should provisionally be monitored in the group of DSP toxins.

### II - Pectenotoxins

Toxicological studies after i.p. administration of Pectenotoxins (PTX) demonstrate a hepatotoxic activity. Diarrheagenic activity of PTX has been described after oral administration *of a higher dose*. However additional data are still lacking regarding the precise mechanism of action of these toxins and the effects of PTX after oral administration. No cases of human intoxication by PTX have been reported. PTX is of *limited* availability due to the lack of standard material and to the difficulty in culturing the producing organism.

*In view of* the co-occurrence in nature of PTX with other toxins of the okadaic acid group, it is difficult to assess the potential toxic effect of PTX. Studies *of the experimental oral toxicity of PTX* are needed in order to understand *itse* real importance. Until more data *are* available, they should be monitored at the same level as OA group toxins.

### III - Okadaic Acid

According to the data on the tumor promotion activity of OA there is no evidence that there may be *a risk a* of long term hazard for shellfish consumers under the current regulations regarding diarrheagenic substances. There is *no good evidence of* the genotoxic potential of OA. Only the diarrheagenic activity of OA should be matter of concern to establish regulatory limits for these toxins. The present protection level of 16 µg OA eq/100 g, agreed by the EU-NRLs group should provisionally be maintained *as it appeared to offer sufficient protection to consumers.*



II

Gerard McCurdy is Programme (rol) Random Bay/Bay or Rollis?

Andrew K Scallop wild fisheries - sampling off individual vessels Grid method  
open/closed/restricted fish logbooks

Cultured Scallops - Direct sampling Set sampling points.

Other shellfish Harvesting areas  
discrete bays - Some subdivided  
- Brotox + Micros for  
Classification  
MAPS - Dept / BIM / MI  
consultative meetings connections  
on species

Andrew K Given the variation between Bioassays in various NREs  
are Bioassays a complete test or are other  
methods more likely reference methods.

Marisa Chemical tests need all standards  
Functional Assays measuring biological activity  
Screen Assay to replace mouse should be a Functional  
Assay.

AZP

Assessment → "In my opinion HP Assay is better because  
you are dealing with smaller amount of tissue  
and when 24 hr result is positive look at  
chemistry"

@ 0.1 ug/g mouse may miss these levels  
because 0.5 seems to be

IV- The Panel did not discuss details of the mouse bioassay for DSPs, as that was outside its terms of reference. It was aware that differences in the preparative techniques did affect the toxins detected, and it was also aware that the present system had not been optimised for the detection and assay of diarrheagenic and non-diarrheagenic toxins. A panel of experts should be able to evaluate the bioassays and other techniques as tools protecting the public.

V- It was recommended that the EC be asked to consider funding the necessary research into the toxicity of the various toxins considered.

VI - General consideration

Future studies on these toxins should not only respond to fundamental toxicology questions but have also as a major goal the assessment of human intoxications and the improvement of public health.



(1)

Richie Consumption without apparent illness

Marissa Toxin may act in other ways than diarrhoea production

Liz Toxin 10 times less than OA.

AZP  $\rightarrow$  Nannogram levels

DSP  $\rightarrow$  8ug

Garry McCurdy Gross underestimation of food poisoning

Marissa 150000 cases of Diarrhoea in France are analysed by French doctors  
1/2 are down to microbiological  
others are unknown

Wayne Acute vs Chronic. If toxin effects since then it must be evaluated from public health. <sup>Absence</sup> of Acute illness must not be overplayed.

Richie How are other countries dealing with Yersin toxins  
24 hour test is not enforced in other countries

Marissa . Even if they are not observed in 8 countries that use 24 hr test... even shorter times are essentially monitoring Yersin toxin

France 24 hr test has been recommended

Spain 3/4 Production Areas use 24 hr test other use 12 test

Finian who is going to do toxicology

100ug/kg IP	500ug
10 ug Cardiac	
2.5ug Marquid	
1 ug No Effect	



**MARINE INSTITUTE**  
FORAS NA hANA

**Marine Institute**

80 Harcourt Street

Dublin 2, Ireland

Ph: +353.1.478 0333

Fx: +353.1.478 4988

Web: [www.marine.ie](http://www.marine.ie)

E-mail: [institute.mail@marine.ie](mailto:institute.mail@marine.ie)